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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> UNGULATE PREBLASTOCYST DERIVED EMBRYONIC STEM CELLS AND USE THEREOF TO PRODUCE CLONED TRANSGENIC AND CHIMERIC UNGULATES  <b>(57) Abstract:</b>  Totipotent ungulate embryonic stem cell lines, e.g., of bovine origin, derived from preblastocyst precursor cells are taught. These cell lines are used for cloning of ungulates, for the production of transgenic cell lines, and for the production of transgenic and chimeric ungulates.		

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**UNGULATE PREBLASTOCYST DERIVED EMBRYONIC STEM  
CELLS AND USE THEREOF TO PRODUCE CLONED TRANSGENIC  
AND CHIMERIC UNGULATES**

Field of the Invention

5

The present invention is generally directed to embryonic stem cell cultures derived from ungulate preblastocyst stage ungulate embryos, preferably bovine preblastocyst stage embryos, methods for  
10 producing said embryonic stem cell cultures, methods of using the embryonic stem cells contained in said cultures for nuclear transplantation of recipient oocytes, methods of using the embryonic stem cells contained in said cultures to produce transgenic  
15 embryonic stem cells, methods of using said transgenic embryonic stem cells for nuclear transplantation in recipient oocytes for the production of transgenic embryos, methods of using the resultant transgenic embryos to produce  
20 transgenic ungulate animals, and transgenic embryonic stem cell cultures and transgenic ungulate animals produced using transgenic preblastocyst derived embryonic stem cell lines.

25 Background of the Invention

Embryo multiplication by nuclear transfer involves the transplantation of living nuclei from embryonic cells, or the whole embryonic cells themselves, into  
30 recipient cells, typically unfertilized eggs, followed by the fusion of the donor and recipient. Such transfers are typically made in order to increase the number of genetically identical embryos which can be obtained from elite genetic stock.

35 The earliest research relating to nuclear transfer of nuclei of embryonic cells into unfertilized eggs

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was performed in amphibians. Specifically, embryonic frog blastomere cells were separated and the nuclear material was reintroduced into frogs' eggs which had been enucleated. See "Transplantation of Living Nuclei from Blastula Cells into Enucleated Frog Egg", Briggs, R. et al., Proc. Natl. Acad. Sci., 38, 455-463, 1972. Subsequent to this research, other experiments were performed in amphibians and amphibian eggs to determine if nuclear material from adult frog somatic or germinal cells could be transplanted into eggs and if the egg would develop into a normal larva. See "Development and Chromosomal Constitution of Nuclear-Transplants Derived from Male Germ Cells," Berradino, MA, et al., J. Exp. Zool., 176, 61-72, 1981.

Transplantation of mammalian living nuclear material into recipient oocytes has also been reported in the literature. The earliest successful reports of nuclear transplantation in higher mammals were achieved from sheep embryos wherein individual blastomeres from 8 and 16 cell embryos were used as the nuclear donors in enucleated or nucleated halves of unfertilized eggs. See "Nuclear Transplantation in Sheep Embryos," Willadsen, S.M. et al., Nature, 320, 63-65, 1986. About the same time, nuclear transplantation of bovine nuclear material was also reported in the literature in "Nuclear Transplantation in Bovine", Robl, J. et al., Theriogenology, 25, 1, 1986. However, the resultant embryos only developed for 43 days out of a normal nine month gestation period.

Fairly recently, substantial improvements in nuclear transplantation methods have been reported in the literature. Many of these improvements have been in the area of enhancing the number of viable embryos

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which may be obtained from a single fertilized embryo.

Essentially, once a fertilized embryo has reached a cleavage stage, which simply means that the embryo comprises at least two cells, it becomes feasible to transfer the nuclei from the cells, or the entire cells themselves, into recipient oocytes which have been enucleated, to thereby create multiple genetically identical embryos from such fusions. By allowing each of the fused nuclear transfer embryos to develop to a multi-cell stage, and then repeating the nuclear transplantation procedure, it is possible to obtain a number of genetically identical nuclear transfer embryos from a single original donor embryo.

Typically blastomeres isolated from pregastrulation embryos comprise the source of such donor nuclei. However, other known sources of donor nuclei include embryonic stem cells.

An inherent limitation in the commercial use of nuclear transfer processes, as practiced to date, arises from the fact that there exist various inefficiencies in the nuclear transfer process. Most especially, not all of the nuclear fusions result in viable embryos. Also, not all of the embryos produced by nuclear fusion are capable of creating a viable (full term) pregnancy in a surrogate animal. This is evidenced, e.g., by the Robl et al. (Id.) reference discussed supra. Accordingly, substantial effort is currently being directed towards optimizing nuclear transfer techniques at each step of the process, so as to make the overall procedure more economically practical and reproducible.

Two patents which are illustrative of recent improvement in nuclear transplantation methods are

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U.S. Patent No. 4,994,384 to Prather et al. and U.S. Patent No. 5,057,042 by Massey.

Both of these patents relate to nuclear transplantation methods which enable the serial  
5 production of many genetically identical bovine animals. In these nuclear transplantation methods, oocytes are recovered from the ovaries or reproductive tracts of cows. These oocytes are then selected for a particular stage of development, and  
10 are enucleated by physical aspiration through a transfer pipette, resulting in an enucleated oocyte which still retains its external membranes and which functions as a recipient donor nucleus or as a donor embryonic cell. Synchronously, a donor embryo at the  
15 proper stage of development, typically at the cleavage or morula stage, is manipulated so that one or more cells or blastomeres are removed from the embryo. The donor cell, which, of course, contains its nucleus, is then inserted into the perivitelline  
20 space of a recipient oocyte. An electronic pulse is then applied in order to fuse the membranes of the donor cell and the recipient oocyte, thus creating an activated, fused single cell embryo.

The resultant single cell nuclear transfer embryo  
25 is then cultured in vitro, or in the oviduct of a mammal, until it reaches a stage of development wherein it may successfully be implanted into a recipient cow. This methodology results in a significant number of fused embryos which are viable,  
30 and which when transplanted surgically or non-surgically into the uteri of cows result in viable pregnancies and the production of genetically identical calves.

Since the basic nuclear transplantable techniques  
35 were set forth in Prather et al. (Id.) and

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Massey (Id.), various aspects of nuclear transplantation methods have been modified or enhanced.

For instance, much is now known about  
5 synchronizing and optimizing the cell cycle stage of the donor and recipient cells prior to fusion. Also, culture methods for fused nuclear transplant embryos may now proceed for longer periods of time in vitro  
prior to implantation into maternal animals. See  
10 e.g., Bondioli et al., "Bovine Nuclear Transplantation", International Application No. PCT/US 88/01906, International Publication No. WO 88/09816, December 15, 1988; and Bondioli et al., Theriogenology, 33, 165-174, 1990 which teach  
15 development of bovine embryos to the morula or blastocyst stage in ligated sheep oviduct prior to transfer to maternal recipients, and Bondioli et al., 1990, (Id.) and Bondioli et al., "Bovine Embryo In Vitro Culture", International Publication No. WO  
20 89/07135, (August 10, 1989); which relate to in vitro culture systems for nuclear transfer embryos. Further, cell-free embryo culture which overcomes the 8-16 cell developmental block has been reported in U.S. patent No. 5,096,822 to Rosenkratz, Jr. et al.

25 Along similar lines, much effort has recently been directed toward maximizing the available supply of genetically identical donor nuclei (or nucleated donor cells) for use in nuclear transfer and for other genetic manipulations.

30 Originally, donor nuclei were obtained directly from embryo cells. However, an inherent limitation to this strategy is that the maximum number of cells available from each embryo is small, on the order of 32-64 cells. It therefore has been the aim of  
35 researchers in this field to maximize the number of



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donor cells from each embryo. This, of course, increases the likelihood of obtaining more genetically identical animals from a particular embryo.

5        Beyond a certain developmental stage it is no longer possible to use every embryo cell for nuclear transplantation because some of the cells are no longer totipotent. This occurs essentially because some of the embryonic cells in the embryo have  
10        undergone significant cellular differentiation. By contrast, totipotent cells are cells which have not as yet significantly differentiated and thus may be used as donor nuclei or nucleated donor cells in nuclear transplantation. For example, it has been  
15        shown that cells of the inner cell mass (ICM), but not trophoblasts, of blastocyst stage bovine embryos (embryo of at least about 64 cells) may be used as donors for nuclear transfer.

20        Given the loss of totipotency which starts at about 64 cells into embryo development, much effort has been directed toward producing continuous cultures of totipotent embryonic cell lines which, under proper growth conditions, remain both totipotent and undifferentiated until these cell  
25        lines are induced to differentiate. For example, such stem cell lines have been isolated from mice, and have putatively been obtained from bovine, ovine, hamster, mink and porcine blastocyst ICM cells. (See, e.g., Doetschman et al., "Establishment of  
30        hamster blastocyst-derived embryonic stem cells", Devel. Biol., 127, 224-227, 1988; Eistetter, "Pluripotent embryonal stem cells can be established from disaggregated mouse morulae". Devel. Growth and Diff. 31, 275-282, 1989; Flechon et al.,  
35        "Characterization of ovine and porcine embryonic stem

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cells", JRF, 40, 25, 1990; Matsui et al., "Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture", Cell, 70, 69, 891-897, 1992; Notarianni et al., "Maintenance and differentiation in culture of pluripotent embryonic cell lines from pig blastocysts", J. Reprod. Fert Suppl., 43, 255-260, 1990; Sukoyan et al., "Isolation and culturization of blastocyst-derived stem cell lines from American mink", Mol. Reprod. Devel., 33, 418-437, 1992.

In bovines, calves have been obtained by nuclear transfer techniques using blastocyst-derived inner cell mass and cultured inner cell mass cells as nuclear donors. (Sims et al., "Production of fetuses from totipotent cultured bovine inner cell mass cells"; Theriogenology, 34, 313, 1993). In sheep, a possible pregnancy has been reported which remained viable 45 days from gestation.

Although embryo development differs from species to species, there exist noteworthy similarities across different types of animal embryos. For example, all ungulate embryos develop at roughly the same rate, which is notably slower than that of mice embryos.

In ungulates, the blastocyst inner cell mass appears after the first round of differentiation which distinguishes the ICM cells from the cells of the trophectoderm. The cells of the trophectoderm, which constitute the majority of blastocyst cells, are not totipotent. Therefore, when preparing ICM - derived bovine stem cells, it is necessary to remove or otherwise separate the inner cell mass cells from the trophectoderm. Unfortunately, it has proven to be relatively difficult to separate the ICM from the trophectoderm. Moreover, and more significantly, it

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has proven to be very difficult to dissociate the cells of the ICM prior to culturing.

To date, there has not been reported in the art a culture of totipotent ungulate embryonic stem cells wherein the cells are derived from an undifferentiated ungulate embryo, as opposed to an ungulate embryo which has already undergone differentiation and which therefore contains cells which have already committed to a particular lineage.

This would be desirable from at least several standpoints. Cells obtained from undifferentiated embryos (preblastocyst stage) are larger than embryonic cells obtained from the inner cell mass. Therefore, they may be easier to manipulate in nuclear transfer processes. Also, the use of embryonic cells from undifferentiated embryos for the production of embryonic stem cell lines would obviate the need to separate totipotent cells (inner cell mass) from differentiated cells (trophectoderm).

The discussion thus far has stressed the importance of embryonic stem cells for the production of cloned animals which are genetically identical. This, of course, is of great importance, especially in the agricultural industry for the production of identical animals having specific desirable traits, e.g., enhanced milk production or increased size. However, another important application of embryonic stem cell cultures is for the production of transgenic embryonic cell lines. Such transgenic cells lines may be used for in vitro research, or may be used as nuclear or cell donors for nuclear transfer to obtain transgenic embryos which, in turn,

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may be used for implantation into maternal recipients to produce transgenic animals.

Transgenic animals have tremendous research and commercial potential. For example, animals may be produced which contain disease causing genes and used as animal models for drug research. Also, transgenic animals may be produced which contain genes providing for desirable traits, e.g., growth hormone genes.

The production of transgenic animals, and in particular transgenic ungulates has been reported in the literature. For example, Simons et al., Bio/Technology, 6, 179-183, 1988 reports the production of transgenic sheep by a microinjection technique. Also, Rexroad et al., Mol. Reprod. Dev., 1, 164-169, 1989 reports the integration of growth-regulating genes in sheep by microinjection of pronuclei.

Also, Biery et al., Theriogenology, 29(1), 224, 1988 discuss the use of pronuclei injection of DNA into bovine zygotes; and McEvoy et al., Theriogenology, 33(4), 819-828, 1990, report the microinjection of very early stage embryos (one and two cell containing bovine ova) to create transgenic cattle. Further, Wilmut et al., Theriogenology, 33(1), 113-123, 1990 report gene transfer in order to modify milk production by microinjection of bovine pronuclei with foreign DNA. The reference also discusses retroviral and stem cell transfer to blastocysts, and the use of sperm containing foreign DNA to fertilize eggs.

A significantly improved method to produce transgenic nonhuman animals, in particular bovines, entails microinjection of embryonic cells with desired genetic material, e.g., a homologous or heterologous DNA, and then use of the injected cells

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as nuclear donors for enucleated eggs. The injected cell and egg are fused, cultured to a requisite stage of development, and then implanted in a maternal animal for the production of genetically transformed animals. This method has been reported to provide for much greater efficiencies than pronuclear injection. Hill et al., Theriogenology, 37, 222, 1992, and Bondioli et al., "Transgenic Animals", First, N. and Haseltine F. (eds.), Butterworth-Heinemann, Stoncham, MA., pp. 265-273, 1991.

See also a recent review article pertaining to the production of transgenic farm animals. Pursel et al., "Status of Research with Transgenic Farm Animals", J. Animal Sci., 71, 10-19, 1993.

#### Object of the Invention

It is an object of the invention to produce in vitro cultures containing totipotent embryonic stem cells derived from an ungulate preblastocyst stage embryo cell.

It is a more specific object of the present invention to produce in vitro cultures containing totipotent bovine embryonic stem cells derived from a bovine preblastocyst stage embryo cell.

It is another object of the invention to provide a method for producing in vitro cultures containing totipotent ungulate embryonic stem cells comprising; (i) removing the zona pellucida from a preblastocyst stage ungulate embryo; (ii) disaggregating the cells of said preblastocyst stage embryo; and (iii) culturing the resultant disaggregated blastomeres in a cell culture medium under culturing conditions which prevent differentiation until embryonic stem cell colonies are obtained.

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It is another object of the invention to provide a method for the continuous production of in vitro cultures containing totipotent ungulate embryonic stem cells comprising; (i) removing the zona  
5 pellucida from a preblastocyst stage ungulate embryo; (ii) disaggregating the cells of said preblastocyst stage embryo; (iii) culturing the resultant disaggregated blastomeres in a cell culture medium under culturing conditions which prevent  
10 differentiation until stem cell colonies arise; and (iv) passaging the individual embryonic stem cells onto a new culture medium which prevents differentiation and provides for the growth of said embryonic stem cells and the production of embryonic  
15 stem cell colonies.

It is a more specific object of the invention to prepare in vitro cultures containing totipotent ungulate embryonic stem cells from a preblastocyst stage embryo; comprising: (i) removing the zona  
20 pellucida from a preblastocyst stage embryo; (ii) disaggregating the cells of the preblastocyst stage embryo; and (iii) co-culturing the resultant disaggregated blastomeres with an established cell feeder layer under conditions which provide for  
25 embryonic stem cell monolayers.

It is another specific object of the invention to provide a method for the continuous production of in vitro cultures of totipotent ungulate embryonic stem cells from a preblastocyst stage embryo cell,  
30 comprising the steps of: (i) removing the zona pellucida from a preblastocyst stage embryo; (ii) disaggregating the cells of the preblastocyst stage embryo; (iii) co-culturing the resultant disaggregated blastomere with an established cell  
35 feeder layer until an embryonic stem cell monolayer

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is obtained; and (iv) passaging the resultant individual embryonic stem cells onto a new cell feeder layer.

5 It is another object of the invention to provide a novel method for nuclear transplantation in ungulates comprising introducing into a recipient ungulate oocyte a totipotent embryonic stem cell derived from an embryo cell of an ungulate preblastocyst stage embryo.

10 It is a more specific object of the invention to provide a novel method for nuclear transplantation in bovines comprising introducing into a recipient bovine oocyte a totipotent embryonic stem cell derived from a embryo cell of a bovine preblastocyst stage embryo.

15 It is another object of the invention to provide a method for nuclear transplantation in ungulates wherein the donor cell introduced into a recipient oocyte comprises an embryonic stem cell prepared by a method comprising the steps of: (i) removing the zona pellucida from a preblastocyst stage embryo;  
20 (ii) disaggregating the cells of the preblastocyst stage embryo; and, (iii) culturing the resultant disaggregated blastomere in a culture medium under conditions which provide for the formation of  
25 embryonic stem cell colonies.

It is another object of the invention to provide a method for nuclear transplantation in ungulates wherein the donor cell introduced into a recipient  
30 oocyte comprises an embryonic stem cell prepared by a method comprising the steps of: (i) removing the zona pellucida from a preblastocyst-stage embryo;  
(ii) disaggregating the cells of the preblastocyst-stage embryo; and (iii) co-culturing the resultant  
35 disaggregated blastomere with an established cell

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feeder layer under conditions which provide for the formation of an embryonic stem cell monolayer.

It is another object of the invention to provide for the production of transgenic embryonic stem cells wherein the improvement comprises transfecting  
5 totipotent embryonic stem cells derived from an ungulate preblastocyst-stage embryo cell with a desired polynucleotide.

It is a more specific object of the invention to provide for the production of transgenic bovine  
10 embryonic stem cells comprising transfecting totipotent embryonic stem cells derived from a bovine preblastocyst-stage embryo cell with a desired polynucleotide.

It is another object of the invention to provide a novel method for producing transgenic ungulate animals comprising: (i) introducing into a recipient ungulate oocyte a totipotent transgenic embryonic stem cell derived from an embryo cell of an ungulate preblastocyst-stage embryo; (ii) fusing the membranes  
20 of the donor cell and the recipient oocyte; (iii) culturing the resultant fused embryo either in vitro or in vivo until it develops to such extent that it can be implanted into a maternal recipient ungulate; and, (iv) implanting the cultured embryo into a  
25 recipient ungulate.

It is a more specific object of the invention to provide a novel method for producing transgenic bovines comprising: (i) introducing into a recipient bovine oocyte a totipotent transgenic bovine embryonic stem cell derived from a preblastocyst stage bovine embryo; (ii) fusing the membranes of the donor cell and the recipient oocyte; (iii) culturing the resultant bovine embryo either in in vitro or in  
30 in vivo until it develops to such extent that it may be  
35



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implanted into a recipient bovine maternal recipient;  
and (iv) implanting the transgenic bovine embryo into  
a maternal bovine recipient.

5 It is another object of the invention to provide  
for the production of an ungulate chimeric embryo  
comprising introducing one or more embryonic stem  
cells derived from an ungulate preblastocyst stage  
embryo cell, which embryonic stem cells may or may  
not comprise a heterologous DNA, into an early stage  
10 embryo, so as to produce a chimeric embryo which  
expresses the genotype of the embryonic stem cells in  
some or all of its cells.

#### Definitions

15

For the purpose of this invention, the following  
terms will be defined as follows:

20 Cleavage embryo refers to an embryo having at least  
two cells which may be disaggregated into individual  
cells.

25 Preblastocyst stage embryo will refer to any ungulate  
embryo having less than or equal to about 64 cells,  
which has not as yet differentiated, and does not  
contain an inner cell mass and trophectoderm.

30 Blastocyst stage embryo refer to an embryo having at  
least about 64 cells which comprises a discernable  
inner cell mass and trophectoderm.

35

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Embryonic stem cells refers to cells derived from preblastocyst stage embryos which lack the zona pellucida and which when cultured under appropriate conditions do not differentiate until they are induced to differentiate.

Trophoblast cells refers to specialized cells in the embryo which have flattened out and surround the blastocoel. These cells ultimately become the placental membranes.

Blastocoel refer to a fluid filled cavity formed during the development of the trophoblast cells which varies in size during embryo development.

Inner Cell Mass (ICM) refer to the cells which are compact and form the inner cell mass of the blastocyst which ultimately become the fetus.

Vitelline Membrane refers to the membrane which surrounds the cytoplasm of the individual cells.

Perivitelline Space is the space located between the vitelline membrane and the zona pellucida.

Zona pellucida refer to the protective covering of the embryo which provides for the aggregation of early embryonic cells.

Zygote refers to the one cell embryo which is surrounded by a smooth vitelline membrane.

Early Morula refers to the embryo stage having approximately 32 cells. The cells are adhered together to provide a compact cell mass.

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Late Morula refers to the embryo, at about 64 cells into embryo development. The cells at that stage have adhered into a tightly compacted mass, having a distinguishable scalloped edge that appears on the outside of the cell mass and exhibits a dappled cytoplasmic appearance.

Feeder Cells refers to any cells which prevent the differentiation of blastomeres and enable the formation of stem cells colonies. In the preferred embodiment these cells will comprise fibroblast cells.

Blastomeres refers to cells obtained from preblastocyst embryos which are cultured for the production of embryonic stem cell colonies.

#### Brief Description Of The Figures

Figure 1 illustrates eight- to sixteen-cell-stage blastomeres (cells) which have just been placed underneath a mouse fibroblast feeder layer.

Figure 2 illustrates eight-to sixteen-cell-stage blastomeres (cells) 24 hours after they had been placed underneath a mouse fibroblast feeder layer. The Figure also illustrates the much larger cytoplasmic volume in comparison with the nuclear volume of the blastomeres.

Figure 3 illustrates an initial ES-cell colony derived from a preblastocyst-stage embryo and demonstrates that the cells of the colony are considerably smaller in size than the blastomeres shown in Figures 1 and 2.

Figure 4 illustrates a blastomere that degenerated and was unsuitable for producing an ES cell line.

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Figure 5 illustrates endoderm-like cells which are generated from blastomeres and undesirable for ES-cell-line production.

5 Figure 6 illustrates embryonic stem cells which were produced from preblastocyst-stage embryos. The Figure demonstrates that the ES cells grow as a monolayer on top of the feeder layer and are epithelial-like in appearance. The Figure demonstrates further that, unlike in blastomeres, 10 most of ES cell volume is nuclear instead of cytoplasmic and there are nucleoli in ES cells.

15 Detailed Description of the Preferred Embodiments of the Invention

The present invention is directed to a culture of ungulate embryonic stem (ES) cells derived from preblastocyst stage embryo cells. In the preferred 20 embodiment these cells will be obtained from bovine preblastocyst stage embryos. However, the use of any preblastocyst ungulate embryo cells is within the scope of the present invention. For example, suitable ungulates include, e.g., cows, sheep, deer, 25 pigs, horses, goats, antelope, etc.

The cells which are to be used as progenitors of the subject ES cells lines will comprise cells derived from an ungulate embryo, e.g., a bovine embryo, that has not as yet reached the blastocyst 30 developmental stage. At this stage, all of the embryo cells are totipotent. Therefore, the invention embraces embryos up to about the early to late morula stage which immediately precedes the blastocyst stage.

35 Ungulate embryos as a group differentiate at a similar rate. Generally, the blastocyst stage of development occurs at about 64 cells into embryo

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development. The late morula stage, which is the stage which precedes the blastocyst stage and comprises about 64 cells. The subject invention accordingly embraces ES cells derived from any  
5 preblastocyst embryo of less than or equal to about 64 cells.

A blastocyst stage embryo has undergone significant differentiation. For example, it comprises an inner cell mass (ICM) of differentiated  
10 trophectoderm cells surrounding a liquid-filled blastocyst. The trophectoderm cells have differentiated to such extent that they are not totipotent. Consequently, such cells are not a suitable source of ES cells. By contrast, the ICM  
15 cells (which eventually give rise to the fetus) are totipotent and may be used to produce ES cells.

As discussed previously, blastocyst stage embryos are disadvantageous sources of ES cells since they comprise both differentiated and non-differentiated  
20 (totipotent) cells which necessitates the separation of these cells (specifically separation of ICM from the trophectoderm) and the dissociation of the ICM prior to culturing which steps have proven to be relatively difficult. By contrast, preblastocyst  
25 stage embryos only comprise totipotent cells; therefore, there is no need to separate totipotent from differentiated cells. Thus, the present invention provides an improvement over previous methods for generating ES cell lines since there is  
30 no risk that the ES cultures will be contaminated by differentiated cells from the trophectoderm, which cells may not be used to produce ES cells.

Preblastocyst cells, e.g., bovine preblastocyst cells are not only functionally distinct from  
35 blastocyst cells (in that they are all totipotent);

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they are morphologically distinct as well. For example, preblastocyst embryo cells are easier to dissociate than blastocyst (ICM) cells. Moreover, given their larger size, they may be easier to manipulate in culture than blastocyst-derived ES cells.

Cells that have the above-noted characteristics may be isolated from any ungulate preblastocyst stage embryo, including, but not limited to, naturally occurring in vivo fertilized cultured embryos, embryos derived from an earlier nuclear transfer procedure, or transgenic embryos derived from an earlier transfection procedure.

The developmental rate of bovine embryos, has been well characterized, particularly with respect to size, shape, and generalized appearance. See, for example, Dorn, C.G. and D.C. Kraemer, Bovine Embryo Grading, 1982, Texas, A. & M. University, College of Veterinary Medicine, Department of Physiology and Pharmacology, College Station, Texas.

Therefore, one of ordinary skill in the art can, without additional guidance, select a suitable undifferentiated, preblastocyst stage embryo for use in the present invention as a source of ES cells.

The present inventors have successfully produced ES cell lines using blastomeres derived from preblastocyst embryos having as few as eight cells to as great as about 64 cells. The ES efficiency, which is defined as the number of ES cell lines established relative to the number of embryos used to produce ES cell lines, appears to vary depending upon the number of cells contained in the preblastocyst embryo used as a source of blastomeres.

For example, embryos having 8-16 cells provided for greater ES efficiencies than did preblastocyst

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embryos having 17-64 cells. Sixteen cell stage embryos appear to provide for the best ES efficiencies.

5 The present inventors also attempted to produce ES cell lines from preblastocyst cleavage stage embryos having only four cells, but have been unsuccessful to date. It is theorized that the development of 4-cell cleavage embryos may still be under the control of maternal message. By contrast, it is theorized that 10 8-cell cleavage embryos, especially late 8-cell cleavage embryos, have developed to a sufficient extent that they develop into ES cell lines autonomously, i.e., in the absence of maternal message.

15 Consequently, it may be possible to obtain ES cells from 4-cell cleavage embryos (and even 2-cell or 1-cell embryos) if the culture medium is supplemented with appropriate maternal factors, e.g. hormones, amniotic fluids, or other factors which 20 mimic maternal embryonic conditions, or if certain factors are eliminated. For example, the subject medium contains glucose which increases ES cell efficiency but may inhibit 4-cell embryos from developing to such extent that ES cells may be 25 obtained.

In the preferred embodiment of the present invention, the preblastocyst stage embryo cells used as a progenitor for ES cells will comprise zygotes which have undergone from about 3 to 6 cell 30 divisions. This corresponds to embryos whose cell number ranges from about 8 to 64 cells. More preferably, preblastocyst embryos which have undergone about 3 to 5 cell divisions will be utilized, and most preferably zygotes which have

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undergone 4 cell divisions and comprise about 16 cells will be utilized.

To prepare an ES cell line from a preblastocyst stage embryo, it is first necessary to remove the zona pellucida which surrounds the embryo, from the embryo. This may be effected by any means which does not adversely affect the viability of the embryo cells. Suitable means include chemical treatment, e.g., with pronase, or mechanical slitting of the zona pellucida to release the embryo.

In the preferred embodiments, the zona pellucida will be removed by treating the preblastocyst stage embryo with pronase at a concentration of about 1 mg/ml for a time ranging from about 1 to 30 minutes.

After the zona pellucida is removed, it is preferable, but not essential to the invention, that the embryo then be placed on a culture medium which produces for reduction of the compact structure of the embryo. This facilitates the later disaggregation of the embryo cells.

Any culture medium which provides for the maintained viability of the embryo and which facilitates reduction of compaction may be used. In the preferred embodiment this culture medium will be MEM- $\alpha$  containing cytochalasin B at 7.5 mg/ml.  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  free medium may also reduce compaction of the embryo. However, such pretreatments are not essential since the embryo may simply be mechanically disaggregated without previous culturing using well known micromanipulation techniques.

Disaggregation of the preblastocyst stage embryo cells will preferably be effected mechanically by repeatedly passing the embryo through a fine bore pipette. This technique, which results in the eventual production of blastomeres, is described in



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Bondioli et al., "Production of Identical Bovine Offspring by Nucleus Transfer", Theriogenology 33(1), 165-174, 1990. Also, treatment of the embryo with a proteolytic enzyme such as trypsin may enhance  
5 disaggregation. However, this may be disadvantageous since proteolytic enzymes may adversely affect the properties of the resultant blastomeres.

After disaggregation is completed (which gives rise to individual cells referred to as blastomeres),  
10 the cells will then be placed in a culture medium which prevents the differentiation of the blastomeres and gives rise to ES cell lines and ES cell colonies.

In the preferred embodiments, the blastomeres will be co-cultured with a feeder cell layer. This feeder  
15 cell layer will comprise any cell layer which provides for the growth of blastomeres and the production of ES cell lines and colonies, but which prevents the differentiation of the cultured blastomeres until later induction. Suitable feeder  
20 cell layers include, e.g., fibroblasts (either primary fibroblasts or fibroblast cell lines) and buffalo rat liver cells.

In the preferred embodiments, the feeder cell layer will comprise fibroblasts. The type of  
25 fibroblasts used does not appear to be essential to the invention. For example, any species fibroblast may be used, these cells may be immortal or normal, and primary cultures or cell lines may be used. STO cells (cells of an immortal embryonic murine  
30 fibroblast cell line which is derived from SIM embryonic fibroblasts and is 6-thioguanine and ouabain resistant), primary mouse fibroblast feeder cell layers, and bovine fibroblast cells have all been proved to be suitable for generating ES cell  
35 lines and colonies.

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It is theorized by the present inventors that co-culturing blastomeres with a feeder cell layer, e.g., a fibroblast feeder layer, prevents differentiation because the feeder cells produce membrane-associated differentiating inhibiting factors, such as LIF (leukemia inhibitor factor). This belief is supported by experimental evidence which indicates that the better the contact between the feeder cell layer and the blastomeres, the better the efficiency of ES cell line generation.

This belief finds further support in the fact that the use of buffalo rat liver cells to prevent the differentiation of mouse ES cells has been reported in the literature. These cells, like fibroblasts, also produce leukemia inhibitor factor (LIF).

Therefore, it should be possible to use other cells as feeder layers which likewise produce differentiating inhibiting factors. The selection of other suitable feeder cells may be effected by one having ordinary skill in the art. Cell layers which provide for the production of ES cell lines and ES colonies may be identified by routine screening, using the methodology described herein, to select for other cell layers which facilitate ES cell line development.

Alternatively, the blastomeres may be cultured in a cell culture medium which contains factors which inhibit differentiation and which enables the production of ES cell lines and ES cell colonies. For example, the blastomeres may be cultured in a LIF containing culture medium, or any other factor which prevents the differentiation of blastomeres.

Preferably, the individual blastomeres will be placed in contact with the feeder layer, most

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preferably fibroblasts. Such feeder cell layers may be produced according to well known methods.

For example, mouse fibroblast feeder layers may be prepared as follows. Mouse fetuses are obtained at 10-20 days of gestation. The head, liver, heart, and alimentary tract are then removed and the remaining tissue washed in phosphate buffered saline and incubated at 37°C in a solution of 0.05% trypsin - 0.02% EDTA.

The mouse cells are then placed in tissue culture flasks containing a culture medium that provides for the support of the feeder layer and the blastomeres. For example, a suitable medium comprises a modified Eagle's Medium containing non-essential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline and serine), ribonucleoside and 2'-deoxyribonucleosides (hereinafter, MEM- $\alpha$ ) supplemented with 100 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, 10% fetal calf serum (FCS) and 0.1 mM  $\beta$ -mercaptoethanol. The plated cells are then cultured until monolayers are produced, preferably at 37°C, 5% CO<sub>2</sub> and 100% humidity.

The invention is not limited to the use of this specific culture medium. For example, one or more of these moieties may be non-essential to the growth of the blastomeres and generation of ES cells. In this regard, it has been shown that the amount of FCS may be reduced to about 5% without detrimental effects to growth of ES cells. However, the available evidence suggests that  $\beta$ -mercaptoethanol may be essential to the culture medium.

After fibroblast cell monolayers are produced, the cells are preferably treated with mitomycin C, e.g., at a concentration of about 10 mg/ml for about three hours. Mitomycin C treatment inhibits DNA synthesis,

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and therefore inhibits cell division of the fibroblasts, yet still provides for the cell layer to support the growth of co-cultured blastomeres.

After formation of a suitable feeder cell layer, e.g., a mouse fibroblast feeder cell layer, or a cell culture medium which provides for the prevention of blastomere differentiation and the formation of ES cell colonies, the blastomeres will be cultured for a sufficient time to provide for the formation of embryonic stem cell colonies.

In the preferred embodiment, this will be effected by placing the preblastocyst derived blastomeres in contact with a fibroblast feeder layer. As discussed supra, it has been found that by providing significant cell-to-cell contact between the blastomeres and feeder layer, it is easier to generate ES cell lines and to prevent the differentiation of the blastomeres. This is theorized to be attributable to membrane associated differentiating inhibiting factors produced by fibroblasts which apparently prevent the differentiation of blastomeres as they develop into ES cell lines. Interestingly, blastomeres do not appear to go through an ICM stage as they multiply into ES cells. This may be another result of the cell-to-cell contact. In the absence of such contact, the preblastocyst derived blastomeres differentiate into trophoblast vesicles.

To maximize the contact between the co-cultured blastomeres and feeder cell layer, e.g., fibroblasts, it is preferable that the blastomeres be placed underneath the feeder layer. However, it is also possible to produce ES cell lines when the blastomeres are placed on top of the cell layer.

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Alternatively, it may be possible to sandwich the blastomeres between two feeder cell layers.

The culture medium will be replaced as necessary, typically every two to three days. After the  
5 blastomeres have been cultured for a requisite period of time, generally on the order of seven to ten days after initiation of culturing, the cells will then be passaged. However, this will vary dependent upon the particular feeder cell layer, the orientation of  
10 cells on the cell layer, the stage of the preblastocyst blastomeres, the composition of the culture medium, among other factors.

The determination as to when passage should be effected will be determined on the basis of when the  
15 cells start to differentiate and exhibit an embryoid-like appearance. The cells will then be passaged to another feeder cell layer or a culture medium which prevents differentiation and provides for the growth of ES cells.

20 Preferably, passage will be effected without chemicals or proteases such as trypsin which may be traumatic to the ES cells. For example, trypsin may denature ES protein and cell receptors. Mechanical means are the preferred means for effecting passage.  
25 For instance, a fine glass needle may be used to cut an ES cell colony from the feeder layer into smaller cell clusters. These clusters may be further broken down by repeated pipetting. Because of the apparently non-degradative nature of this method, the  
30 cells may be passaged at higher dilutions such as 1:100 rather than 1:5 or 1:10. Also, such cells tend to become re-established more rapidly than cells passaged by chemical or enzymatic methods.

The subject ES cells may be passaged indefinitely  
35 using the described methodology, thus providing for

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an essentially unlimited number of ES cells, which, at this stage, may be considered to be a embryonic stem cell line. The present inventors currently have an actively growing ES cell line produced according to the present invention which has been passaged for about 13 months.

As discussed previously, the preblastocyst cells used to produce the subject ES cell lines are functionally and morphologically distinct from blastocyst cells. Also, the ES cell lines produced using preblastocyst progenitors are morphologically different from blastocyst-derived ES cells. For example, preblastocyst derived ES cells are larger, and typically contain more cytoplasm and more lipid vesicles than blastocyst derived ES cells. The doubling time of the ES cells of the present invention ranges from about 24 to 28 hours.

The ungulate ES cells produced according to the invention, e.g., bovine ES cells may be used as donor cells in nuclear transfer protocol such as described in United States Patents 4,994,384 and 5,057,420 to Prather et al. and Massey respectively, or may be used in any other known method of nuclear transfer.

Speaking generally, in a nuclear transfer protocol, an oocyte, typically at metaphase II, is enucleated. A membrane-bound nucleus, typically in the form of a donor cell, is then inserted into the enucleated oocyte. The two cells are then fused, e.g. by electrofusion, PEG fusion, or viral-induced fusion to provide a single-cell nuclear transfer embryo. The nucleus transfer embryo is then cultured in vitro or in vivo to the morula or blastocyst stage according to known methods, at which time it will be implanted into a maternal recipient where it will give rise to a full term fetus.

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In using the ES cells of the invention for nuclear transfer protocols, the ES cells will preferably be disaggregated to provide single cells. This may be effected by chemical or mechanical means, however, mechanical means is again preferred. In addition, a pronase treatment at about 0.1 to 5.0 mg/ml may also be effected to facilitate breaking down the outer layers of the ES cells.

After single ES cells are produced, the individual cells may be incorporated directly as donors into recipient oocytes which have previously been enucleated. Alternatively, the production of donor ES cells and suitable recipient enucleated oocytes may be synchronized before fusion, according to known methods.

The donor and recipient will be fused according to known methods such as are identified supra. The resultant fused nuclear transfer embryos will then be cultured according to known methods to such stage that these embryos may be successfully implanted into maternal recipients, typically to the morula or blastocyst stage. Suitable methods and culture medium for culturing embryos in vitro and in vivo are known to the art, and may be utilized to culture the subject ungulate embryos preferably bovine embryos.

A preferred post-fusion culture medium is exemplified in United States Patent No. 5,096,822 by Rosenkratz, Jr. et al. A preferred post-fusion culture method will comprise culturing the nucleus transfer embryos for about three to four days in CR1 medium supplemented with amino acids (CR1aa) and 3 mg/ml bovine serum albumin (BSA) followed by culturing in CR1aa plus 10% fetal calf serum for about three to four days. CR1aa medium, variations thereof, and use of these medium to culture post-

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fusion embryos is disclosed in U.S. patent No. 5,096,822. However, this medium is only exemplary, many other media and protocols for culturing post-fusion embryos are known and should be suitable for use in the present invention.

After culturing for a requisite time period, the embryos which develop to the blastocyst or morula stage will be transferred to recipient maternal ungulates, e.g., bovines which will give rise to identical ungulate fetuses. Also, in vivo culture may be effected short term in a sheep oviduct prior to implantation as an alternative in vitro culturing.

The subject ES stem cells will also provide materials which may be used for the production of transgenic or genetically altered ES cells, which in turn may be used to produce transgenic or genetically altered ungulates, e.g., bovines.

Methods for introduction of polynucleotides, i.e., desired DNA and/or RNA's into cells in culture are well known in the art. Such methods include, e.g., electroporation, retroviral vector infection, particle acceleration, transfection, and microinjection.

The cells which contain the desired polynucleotide, e.g., a desired gene, which may be homologous or heterologous to the host cell, will be selected according to known methods. For example, the cells may be co-transfected with a marker gene which provides for selection.

Once a culture of transgenic ES cells is obtained which contains a desired polynucleotide integrated in its genome, the individual cells may be used as nuclear transfer donors. This is a particularly advantageous use of the present invention. Thereby, the transgenic ES cell will facilitate the production



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of many identified animals having an identical genetic modification, e.g., a desired gene integration. These animals will provide a means of in vivo study of the effects of the particular gene.

5 Also, animals may be obtained which express a disease causing gene and may be used to assay potential drug therapies for treatment of the particular disease condition.

10 The transfection of ES cells in vitro with a desired polynucleotide will permit most of the genetic modification steps to be performed in vitro. This avoids the need to perform repeated breeding to introduce a desired gene into a particular genetic background. Also, one is able to characterize the  
15 donor cell genome in vitro (e.g., by use of suitable probes) that is impossible when the donor is a primary embryo cell. Consequently, this procedure may be accomplished faster than previous breeding methods. Also since one has a high expectation that  
20 the offspring will express the desired gene, the need to screen animals is substantially reduced. Therefore, the invention should significantly reduce the cost of animal breeding, and the production of transgenic animals, particularly among larger  
25 domesticated animals, which have relatively prolonged gestation periods and which bear only one or a few offspring per pregnancy.

30 Additionally genetically altered preblastocyst embryos may themselves be used as a source of blastomeres and the production of ES cells. Also, these genetically altered ES cells may be subjected to further genetic modifications.

35 Yet another use for the subject preblastocyst-derived ES cells is in the production of chimeric animals. As in the previous case, an ES cell derived

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from preblastocyst stage embryo cells may be genetically altered, as desired, before it is used to repopulate an early stage embryo. After culture in vitro and/or in vivo, such repopulated embryos will  
5 give rise at some frequency to animals which express in some or all of their cells, the genetic material of the inserted genetically altered ES cells used for repopulation.

The present invention will now be further  
10 illustrated by reference to the following examples which are provided solely for purposes of illustration and are not intended to be in any way limitative.

15 EXAMPLE 1

Preblastocyst stage bovine embryos were obtained from in vivo fertilizations and in vitro fertilizations. These embryos ranged in size from  
20 the 8-cell stage to the 64-cell stage, and were selected such that they were between 2 and 5 days from time of fertilization. These embryos were then treated with 1 mg/ml of pronase to remove the zona pellucida. Some of these embryos were then placed in  
25 phosphate buffered saline (PBS) medium containing 7.5  $\mu$ g/ml of cytochalasin B to reduce the compactness of the embryo structure and therefore facilitate the disaggregation of cells. However, some embryos were disaggregated without a cytochalasin B pretreatment.

30 Individual blastomeres, removed from the preblastocyst stage embryos ranging in size from between 8 to 64 cells, were then placed either on top or underneath a mouse fibroblast feeder cell layer prepared according to the method described infra. In  
35 particular, the feeder cell layer was prepared from

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mouse fetuses at ten to twenty days of gestation. The head, liver, heart and alimentary tract were removed from the fetuses and the remaining tissue was washed and incubated at 37°C in 0.05% trypsin-0.02% EDTA. Loose cells were then cultured in tissue culture flasks containing MEM- $\alpha$  supplemented with penicillin, streptomycin, 10% fetal calf serum and 0.1 mM  $\beta$  mercaptoethanol. The feeder cell cultures were established over a two- to three-week culture period at 37°C, 5% CO<sub>2</sub> and at 100% humidity. These cells were then treated with mitomycin C at 10  $\mu$ g/ml prior to their usage as feeder cells.

The mitomycin C-pretreated fibroblast layer was then used as a feeder cell layer for the blastomeres. In some experiments the individual blastomeres were placed on top of the feeder cell layer. However, ES cell lines were more readily established, and differentiation was better inhibited, when the blastomeres were placed beneath the feeder layer. As discussed supra, it is believed that this enhanced cell-to-cell contact provides for the blastomeres to be more in contact with membrane associated differentiating inhibiting factors such as LIF. Cells placed on top of the feeder layer had lesser cell-to-cell contact between the blastomeres and feeder cells. Also, the blastomeres occasionally differentiated into trophoblast vesicles.

Every two to three days, the MEM- $\alpha$  plus 10% FCS growth medium was replaced. After the cells had been cultured for a total of about seven to ten days, embryonic stem cell monolayers were obtained. Also, around this time into the culturing, the blastomeres (which now had become ES cell lines) start to differentiate and exhibit an embryoid-like appearance.

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Accordingly, the cells are passaged at this time onto new feeder layers. This was effected mechanically using a fine glass needle to cut the ES cell monolayer into smaller cell clusters. These cell clusters were then repeatedly pipetted at a 1:100 dilution onto fresh fibroblast feeder layers.

This method has resulted in the generation of numerous ES cell lines from preblastocyst-derived embryos from the 8- to 64-cell stage, and has provided for both male and female ES cell lines.

The ES cell data obtained by the above-described method is presented in Table 1 below. This Table also contains data relating to the use of the subject ES cells as nuclear transfer donors. This use is discussed in Example 2 below.

TABLE 1

## EMBRYONIC STEM CELL DATA

Starting Embryo	No. Starting Embryos	No. ES Cell Lines	Efficiency
Blastocyst	42	22	53%
Morula	11	6	55%
8-16 Cell	12	8	67%
4-cell	30	0	0%
Nuclear Transfer	19	13	68%

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EXAMPLE 2

ES cell lines obtained according to the present invention have also been used as nuclear donors in the nuclear transfer protocols taught by Massey, U.S. Patent, No. 5,057,420, and the Prather et al., U.S. Patent, No. 4,994,384. Before fusion the ES cells were mechanically disaggregated and treated with a low concentration of pronase, between 0.1 and 1.0 mg/ml, to weaken the cell-to-cell contact. The treated ES cells were then placed in the perivitelline space of an enucleated oocyte. The cells were fused by electrofusion, and the resulting nuclear-transfer (NT) embryos were cultured for three to four days in CR1aa plus 3 mg/ml BSA, and then for three to four additional days in CR1aa plus 10% FCS. At the end of the three to eight day culture period, NT embryos that had developed to the blastocyst stage were transferred to recipient females and pregnancies were established. Of the fused embryos (NT embryos), 11/483 developed to the blastocyst stage and 2/11 of the implanted blastocysts resulted in pregnancy. This translates roughly into a 2% blastocyst success rate and a 20% pregnancy success rate.

In order to further illustrate the results obtained by the subject invention, reference may be had to Figures 1-6 of this application. As discussed *supra*, Figures 1 and 2 illustrate 8- to 16-cell stage blastomeres which have been placed underneath a mouse fibroblast feeder layer. Figure 2 in particular illustrates the large differential between nuclear and cytoplasmic volume of the blastomeres.

By contrast, Figure 3 illustrates an initial ES cell colony derived from a preblastocyst-stage embryo. The illustration demonstrates that the ES

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cells of the colony are much smaller than the blastomeres illustrated in Figures 1 and 2.

Figure 4 illustrates a degenerated blastomere which did not result in an ES cell line.

5        Figure 5 illustrates endoderm-like cells generated from blastomeres. These cells are undesirable for ES cell line production.

10        Finally, Figure 6 illustrates ES cells produced from preblastocyst-stage embryos and demonstrates that the ES cells grow as a monolayer which is epithelial in appearance. Also, unlike in blastomeres, the majority of the cell volume of the ES cells is nuclear rather than cytoplasmic and the ES cells comprise nucleoli.

15        It is believed that these figures provide further evidence as to the advantages obtained by the present invention.

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## WHAT IS CLAIMED IS:

1. An in vitro culture of totipotent embryonic stem cells derived from an ungulate preblastocyst stage embryo cell.

2. The in vitro culture of claim 1, wherein the preblastocyst stage embryo cell is derived from a bovine species.

3. The in vitro culture of claim 1, wherein the ungulate preblastocyst stage embryo comprises from about 8 to 64 cells.

4. A co-culture comprising:

- (i) embryonic stem cells derived from an ungulate preblastocyst stage embryo cell; and
- (ii) a feeder layer which provides for the growth of said embryonic stem cells and prevents the differentiation thereof.

5. The co-culture of claim 4, wherein the feeder layer comprises cells which produce a differentiating inhibitor factor.

6. The co-culture of claim 5, wherein said inhibiting factor is leukemia inhibitor factor.

7. The co-culture of claim 6, wherein said feeder cells are fibroblasts or buffalo rat liver cells.

8. The co-culture of claim 4, wherein the preblastocyst stage embryo cell is from a bovine species.

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9. A method for preparing an in vitro culture of totipotent ungulate embryonic stem cells from a preblastocyst stage embryo cell comprising the steps of:

- 5           (i) removing the zona pellucida from a preblastocyst stage embryo;
- (ii) disaggregating the cells of the preblastocyst stage embryo into individual blastomeric cells, and
- 10           (iii) culturing the resultant blastomeres in a culture medium which prevents differentiation of the blastomeres but which permits the formation of embryonic stem cell colonies.

15           10. The method of claim 9, wherein said ungulate embryonic stem cells are from a bovine.

          11. The method of claim 9, wherein the zona pellucida is removed mechanically, chemically or

20           enzymatically.

          12. The method of claim 9, wherein disaggregation of the preblastocyst stage embryo is effected mechanically or enzymatically.

25           13. The method of claim 12, wherein mechanical disaggregation comprises repeated passage of the embryo through a fine bore pipette.

30           14. The method of claim 9, wherein culturing of the blastomeres is effected on a feeder layer.

          15. The method of claim 14, wherein said feeder layer comprises cells which produce a differentiating

35           inhibitory factor.



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16. The method of claim 15, wherein said feeder layer produces leukemia inhibitor factor (LIF).

5 17. The method of claim 14, wherein the feeder layer comprises fibroblasts or buffalo rat liver cells.

10 18. The method of claim 17, wherein the fibroblasts are selected from the group consisting of bovine fibroblasts, primary mouse fibroblasts and STO cells.

15 19. The method of claim 9, wherein culturing is effected in a medium which comprises a differentiating inhibitor factor.

20 20. The method of claim 19, wherein said factor is leukemia inhibitor factor.

20 21. The method of claim 19, wherein such culture medium includes  $\beta$ -mercaptoethanol.

25 22. The method of claim 9, which provides for the continuous production of embryonic stem cells by passaging the resultant embryonic stem cell monolayers into a new culture medium or onto a new feeder layer when the cells start to differentiate and/or to develop an embryoid appearance.

30 23. The method of claim 22, wherein said passaging is effected about every seven to ten days.

35 24. The method of claim 10, wherein prior to disaggregation, the embryo is treated with cytochalasin B.

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25. The method of claim 9, wherein the blastomeres are cultured on top, underneath or between one or more feeder layers.

5        26. A method for nuclear transplantation in ungulates wherein the improvement comprises:  
         introducing into an enucleated recipient oocyte  
         an embryonic stem cell produced using ungulate  
         embryonic cells derived from a preblastocyst stage  
10        embryo.

27. The method of claim 26, wherein the embryonic stem cells are of bovine origin.

15        28. The method of claim 26, wherein the embryonic stem cell is produced by process comprising:

- (i) removing the zona pellucida from a preblastocyst stage embryo;
- 20        (ii) disaggregating the cells of the preblastocyst stage embryo to produce individual blastomeres;
- (iii) culturing said blastomeres in a culture medium which prevents the differentiation thereof,  
25        but which prevents the growth and formation of embryonic stem cell colonies.

29. The method of claim 28, wherein culturing is effected in a co-culture comprising blastomeres  
30        and a feeder cell layer.

30. The method of claim 29, wherein the feeder cell layer comprises fibroblasts or rat buffalo liver cells.

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31. The method of claim 28, wherein the culture medium includes  $\beta$ -mercaptoethanol.

5 32. The method of claim 28, which provides for passaging of the embryonic stem cell colonies when they start to differentiate and/or to develop an embryoid appearance.

10 33. The method of claim 28, wherein the zona pellucida is removed mechanically, chemically or enzymically.

15 34. The method of claim 32, wherein prior to mechanical disaggregation the embryo is treated with cytochalasin B.

20 35. A transgenic embryonic stem cell derived from an ungulate preblastocyst derived embryo cell which comprises integrated in its genome a homologous or heterologous polynucleotide.

36. The transgenic embryonic stem cell of claim 35, wherein said embryo cell is of bovine origin.

25 37. A method of using the transgenic embryonic stem cell of claim 35, for nuclear transplantation comprising introducing said stem cell into a recipient oocyte and fusing the oocyte and embryo stem cell to produce a transgenic ungulate embryo.

30 38. The method of claim 37, wherein said embryo is cultured in vitro or in vivo for a sufficient time prior to implantation into a recipient maternal animal.

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39. The method of claim 37, wherein the embryonic stem cell is of bovine origin.

5 40. The method of claim 38, wherein the embryonic stem cell is of bovine origin and the recipient animal is a bovine.

10 41. A method for producing a chimeric embryo comprising introducing one or more embryonic stem cells derived from preblastocyst stage embryo into an early stage embryo to produce a chimeric embryo which expresses the genotype of the introduced embryonic stem cells in some or all of its cells.

15 42. The method of claim 41, wherein the introduced embryonic stem cells are transgenic.

20 43. The method of claim 41, wherein the early stage embryo is bovine.

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## AMENDED CLAIMS

[received by the International Bureau on 15 May 1995 (15.05.95);  
original claims 1-4, 9, 10, 22, 26, 28, 32, 35 and 41 amended;  
new claims 44 and 45 added; remaining claims unchanged (7 pages)]

1. An in vitro culture of totipotent embryonic stem cells derived from an ungulate preblastocyst stage embryo cell, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs.
2. The in vitro culture of claim 1, wherein the preblastocyst stage embryo cell is derived from bovine embryos.
3. The in vitro culture of claim 1, wherein the ungulate preblastocyst stage embryo cell is derived from a preblastocyst embryo of from about 8 to about 64 cells.
4. A co-culture comprising:
  - (i) embryonic stem cells derived from an ungulate preblastocyst stage embryo cell, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs; and
  - (ii) a feeder layer which provides for the growth of said embryonic stem cells and prevents the differentiation thereof.
5. The co-culture of claim 4, wherein the feeder layer comprises cells which produce a differentiating inhibitor factor.
6. The co-culture of claim 5, wherein said inhibiting factor is leukemia inhibitor factor.
7. The co-culture of claim 6, wherein said feeder cells are fibroblasts or buffalo rat liver cells.

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8. The co-culture of claim 4, wherein the preblastocyst stage embryo cell is from a bovine species.

5           9. A method for preparing an in vitro culture of totipotent embryonic stem cells from an ungulate preblastocyst stage embryo cell, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs, comprising the  
10 steps of:

(i) removing the zona pellucida from a preblastocyst stage embryo;

(ii) disaggregating the cells of the preblastocyst stage embryo into individual  
15 blastomeric cells; and

(iii) culturing the resultant blastomeres in a culture medium which prevents differentiation of the blastomeres but which permits the formation of embryonic stem cell colonies.  
20

10. The method of claim 9, wherein said embryonic stem cells are of bovine origin.

11. The method of claim 9, wherein the zona  
25 pellucida is removed mechanically, chemically or enzymatically.

12. The method of claim 9, wherein  
30 disaggregation of the preblastocyst stage embryo is effected mechanically or enzymatically.

13. The method of claim 12, wherein mechanical  
35 disaggregation comprises repeated passage of the embryo through a fine bore pipette.

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14. The method of claim 9, wherein culturing of the blastomeres is effected on a feeder layer.

5 15. The method of claim 14, wherein said feeder layer comprises cells which produce a differentiating inhibitory factor.

10 16. The method of claim 15, wherein said feeder layer produces leukemia inhibitor factor (LIF).

17. The method of claim 14, wherein the feeder layer comprises fibroblasts or buffalo rat liver cells.

15 18. The method of claim 17, wherein the fibroblasts are selected from the group consisting of bovine fibroblasts, primary mouse fibroblasts and STO cells.

20 19. The method of claim 9, wherein culturing is effected in a medium which comprises a differentiating inhibitor factor.

25 20. The method of claim 19, wherein said factor is leukemia inhibitor factor.

21. The method of claim 19, wherein such culture medium includes  $\beta$ -mercaptoethanol.

30 22. The method of claim 9, which provides for the continuous production of embryonic stem cells by passaging the blastomeres into a new culture medium or onto a new feeder layer when the cells start to exhibit signs of differentiation.

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23. The method of claim 22, wherein said passaging is effected about every seven to ten days.

24. The method of claim 10, wherein prior to  
5 disaggregation, the embryo is treated with cytochalasin B.

25. The method of claim 9, wherein the blastomeres are cultured on top, underneath or  
10 between one or more feeder layers.

26. A method for nuclear transplantation in ungulates wherein the improvement comprises:  
introducing into an enucleated recipient  
15 oocyte an embryonic stem cell produced using embryonic cells derived from an ungulate preblastocyst stage embryo, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs.

20 27. The method of claim 26, wherein the embryonic stem cells are of bovine origin.

28. The method of claim 26, wherein the  
25 embryonic stem cell is produced by process comprising:

- (i) removing the zona pellucida from a preblastocyst stage embryo;
- (ii) disaggregating the cells of the  
30 preblastocyst stage embryo to produce individual blastomeres;
- (iii) culturing said blastomeres in a culture medium which prevents the differentiation thereof, but which permits the growth and formation of  
35 embryonic stem cell colonies.



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29. The method of claim 28, wherein culturing is effected in a co-culture comprising blastomeres and a feeder cell layer.

5 30. The method of claim 29, wherein the feeder cell layer comprises fibroblasts or rat buffalo liver cells.

10 31. The method of claim 28, wherein the culture medium includes  $\beta$ -mercaptoethanol.

15 32. The method of claim 28, which provides for passaging of the embryonic stem cell colonies when they start to exhibit signs of differentiation.

33. The method of claim 28, wherein the zona pellucida is removed mechanically, chemically or enzymically.

20 34. The method of claim 32, wherein prior to mechanical disaggregation the embryo is treated with cytochalasin B.

25 35. A transgenic embryonic stem cell derived from an ungulate preblastocyst stage embryo cell, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs, which comprises a DNA sequence integrated in its genome corresponding to a homologous or heterologous polynucleotide.

30

36. The transgenic embryonic stem cell of claim 35, wherein said embryo cell is of bovine origin.

35

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37. A method of using the transgenic embryonic stem cell of claim 35, for nuclear transplantation comprising introducing said stem cell into a recipient oocyte and fusing the oocyte and embryonic stem cell to produce a transgenic ungulate embryo.

38. The method of claim 37, wherein said embryo is cultured in vitro or in vivo for a sufficient time prior to implantation into a recipient maternal animal.

39. The method of claim 37, wherein the embryonic stem cell is of bovine origin.

40. The method of claim 38, wherein the embryonic stem cell is of bovine origin and the recipient animal is a bovine.

41. A method for producing a chimeric embryo comprising introducing one or more embryonic stem cells derived from an ungulate preblastocyst stage embryo into an early stage embryo to produce a chimeric embryo which expresses the genotype of the introduced embryonic stem cells in some or all of its cells, wherein said ungulate is selected from the group consisting of cows, sheep, goats, horses and pigs.

42. The method of claim 41, wherein the introduced embryonic stem cells are transgenic.

43. The method of claim 41, wherein the early stage embryo is bovine.

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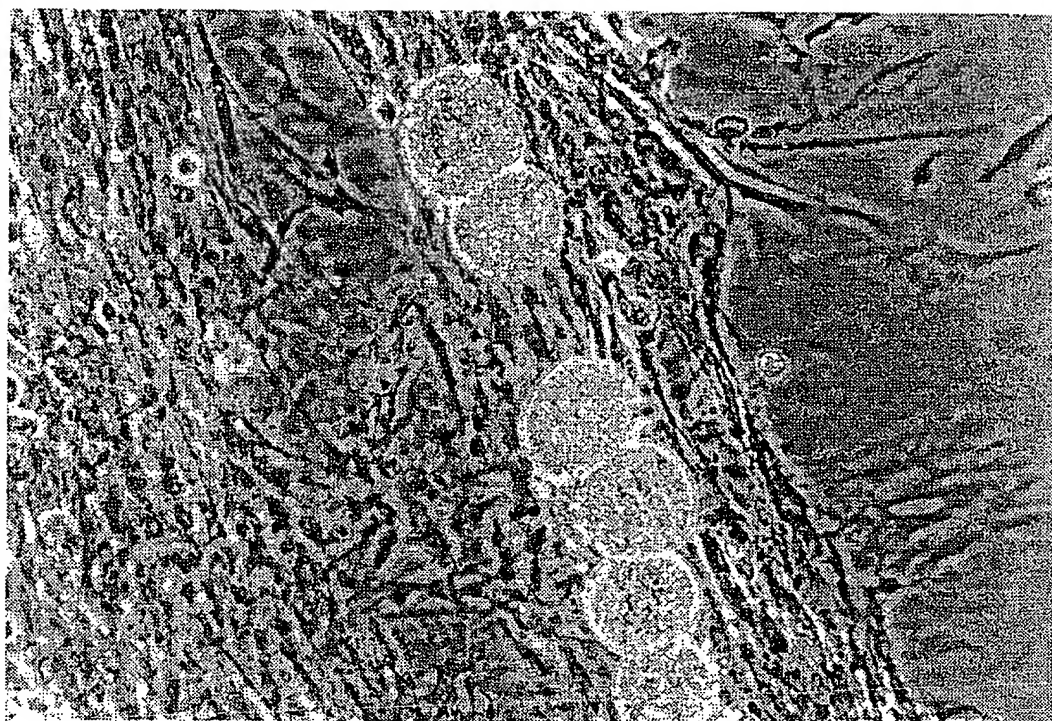
44. The method of claim 22, wherein differentiation is exhibited by the formation of embryoid bodies.

5        45. The method of claim 32, wherein differentiation is exhibited by the formation of embryoid bodies.

10

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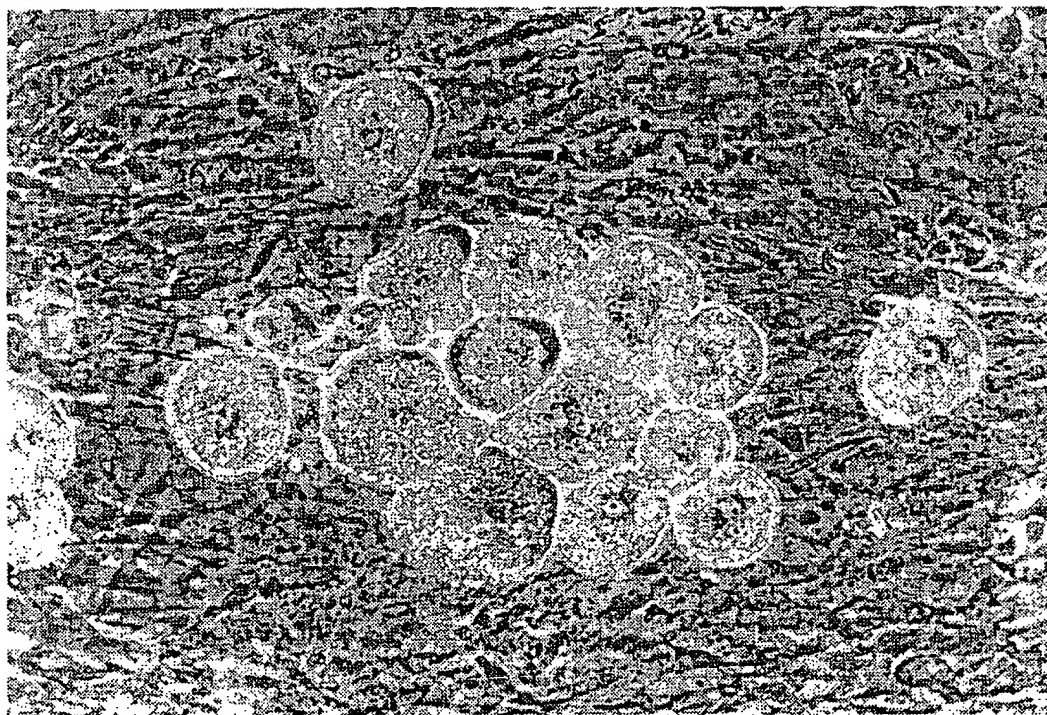
FIG. 1



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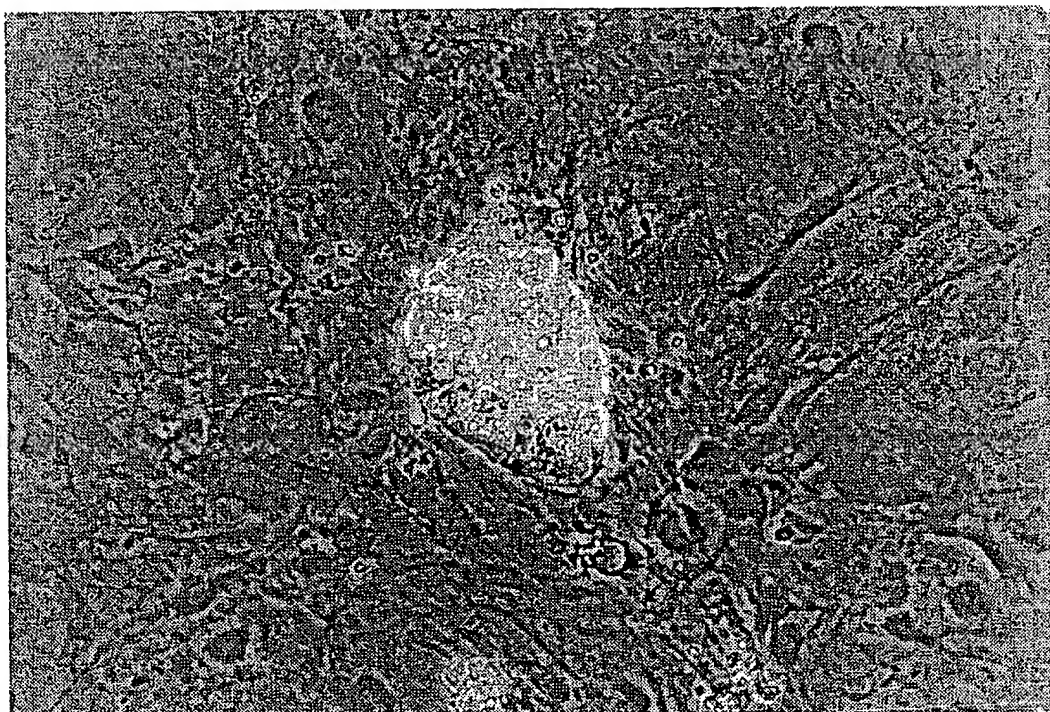
2/6

FIG. 2



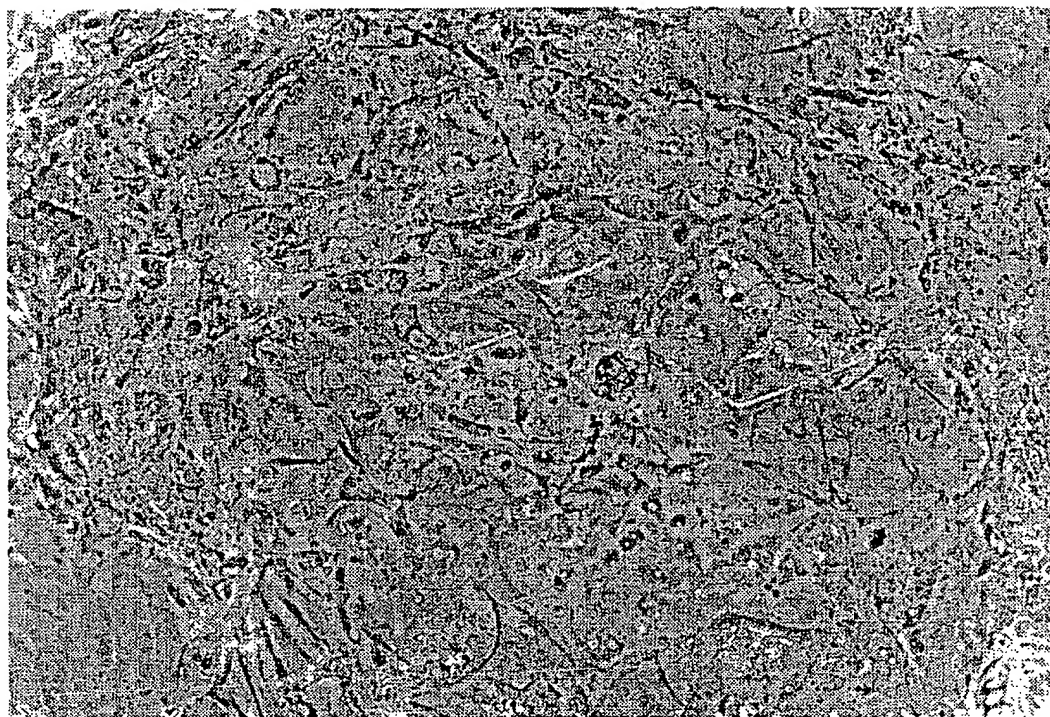
3/6

FIG. 3



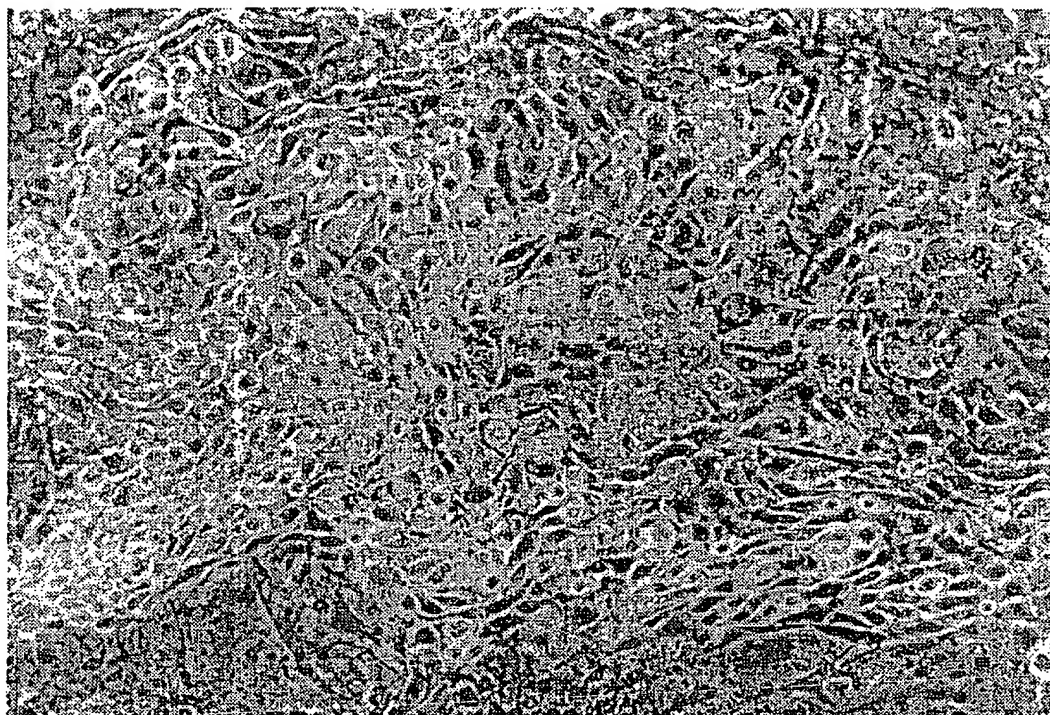
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FIG. 4



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FIG. 5

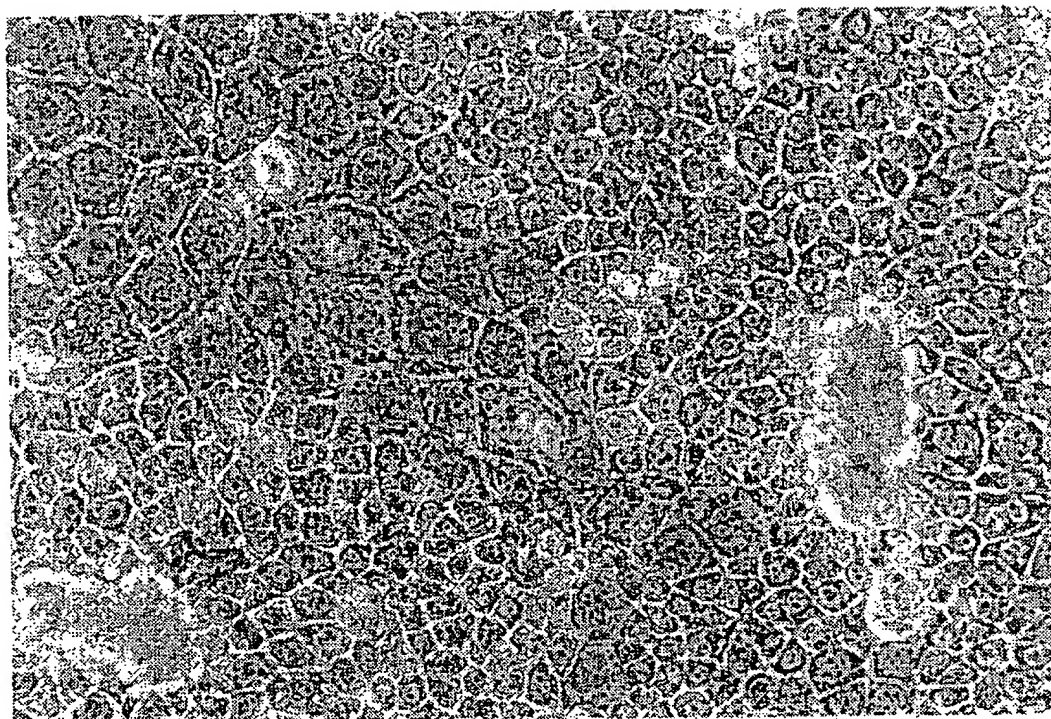


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FIG. 6



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14587

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 5/00, 5/06, 5/10, 15/00, 15/01

US CL : 435/172.3, 240.2, 240.21; 800/2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 240.2, 240.21; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR REPRODUCTION AND DEVELOPMENT, Volume 36, issued 1993, N.C. Talbot et al., "Alkaline Phosphatase staining of Pig and Sheep epiblast cells in culture", pages 139-147, see entire document.	1-43
Y	JOURNAL OF REPRODUCTION AND FERTILITY, Volume 98, issued 1993, T. Ysunoda et al., "Nuclear transplantation of embryonic stem cells in mice", pages 537-540, see entire document.	1-43

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

06 FEBRUARY 1995

Date of mailing of the international search report

13 MAR 1995

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14587

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR REPRODUCTION AND DEVELOPMENT, Volume 36, issued 1993, J.R. Giles et al., "Pluripotency of cultured rabbit inner cell mass cells detected by isozyme analysis and eye pigmentation of fetuses following injection into blastocysts or morulae", pages 130-138, see entire document.	1-43
Y	MOLECULAR REPRODUCTION AND DEVELOPMENT, Volume 36, issued 1993, M.A. Sukoyan et al., "Embryonic stem cells derived from morulae, inner cell mass, and blastocysts of mink: Comparisons of their pluripotencies", pages 148-158, see entire document.	1-43
Y	ROUX'S ARCHIVES IN DEVELOPMENTAL BIOLOGY, Volume 196, issued 1987, A. Handyside et al., "Towards the isolation of embryonal stem cell lines from the sheep", pages 185-190, see entire document.	1-43
Y	DEVELOPMENTAL BIOLOGY, Volume 127, issued 1988, T. Doetschman et al., "Establishment of hamster blastocyst-derived embryonic stem (ES) cells", pages 224-227, see entire document.	1-43
Y	JOURNAL OF REPRODUCTION AND FERTILITY, SUPPLEMENT, Volume 41, issued 1990, E. Notarianni et al., "Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts", pages 51-56, see entire document.	1-43
Y	BIOTECHNOLOGY, Volume 10, issued May 1992, A. Bradley et al., "Modifying the mouse: Design and desire", pages 534-539, see entire document.	1-43
Y	US, A, 5,057,420 (MASSEY) 15 October 1991, see entire document.	1-43
Y	MOLECULAR BIOLOGY OF MEDICINE, Volume 6, issued 1989, H. Baribault et al., "Embryonic stem cell culture and gene targeting in transgenic mice", pages 481-492, see entire document.	1-43
Y	ROUX'S ARCHIVES IN DEVELOPMENTAL BIOLOGY, Volume 201, issued 1992, S. Saito et al., "Bovine embryonic stem cell-like cell lines cultured over several passages", pages 134-140, see entire document.	1-43

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14587

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELL SCIENCE, SUPPLEMENT, Volume 10, issued 1988, J.K. Heath et al., "Regulatory factors of embryonic stem cells", pages 257-266, see entire document.	1-43
Y	THERIOGENOLOGY, Volume 33, number 4, issued April 1990, R.M. Strojek et al., "A method for cultivating morphologically undifferentiated embryonic stem cells from porcine blastocysts", pages 901-913, see entire document.	1-43
Y	THERIOGENOLOGY, Volume 34, number 5, issued November 1990, J. A. Piedrahita et al., "On the isolation of embryonic stem cells: comparative behavior of murine, porcine, and ovine embryos", pages 879-901, see entire document.	1-43
Y	JOURNAL OF REPRODUCTION AND FERTILITY, SUPPLEMENT, Volume 43, issued 1991, E. Notarianni et al., "Derivation of pluripotent, embryonic cell lines from the pig and sheep", pages 255-260, see entire document.	1-43
Y	THERIOGENOLOGY, Volume 39, issued 1993, M.M. Sims et al., "Production of fetuses from totipotent cultured bovine inner cell mass cells", page 313, see entire document.	1-43
Y	THERIOGENOLOGY, Volume 39, issued 1993, N. Strelchenko et al., "Effects of T3-cell line conditioned medium on the formation of embryonic stem cell (ES) like cells in domestic animals", page 319, see entire document.	1-43

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US94/14587

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, CA, Medline, Embase, Biosis, Derwent

Search Terms: Ungulate; embryo?; stem?; cell#; cow; bovine; feeder; leukemia; inhibit?; lif; dif; preblastocyst?; buffalo; brl; mercaptethan?; embryoid; cytochalasin; zona; pellucida; nuclear; transplant?; transgen?